

"LIESEGANG-LIKE" RINGS OF GROWTH AND INHIBITION OF BACTERIA IN AGAR CAUSED BY METAL IONS AND CHELATING AGENTS^{1, 2}

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It was reported in previous studies that inhibitory concentrations of certain chelating or complexing agents did not inhibit several microorganisms when these agents were tested simultaneously (Feeney, 1951; Feeney and Nagy, 1952). Amounts of the metal-binding agents which were strongly inhibitory when tested alone were necessary in order to obtain these mutual preventions of inhibitory action. The mutual preventions did not occur when a metal ion specific for one of the complexing agents was also added. These effects were interpreted as possibly caused by the presence or absence of metal-ion imbalances induced by the complexing agents.

In the present study we have attempted to delineate further the importance of relative concentrations of metal ions and chelating agents. This has been done by producing gradient zones of concentrations of ions and chelating agents by diffusion in agar media. Alternate zones or rings of growth and inhibition have been obtained by varying the concentrations of chelating agents or metal ions in agar plates and by placing filter paper discs containing different concentrations of chelating agents or metal ions on the agar and allowing diffusion to occur before incubation. These alternate zones appear to resemble the Liesegang rings obtained by allowing metal salts to diffuse through gelatin or agar (Gortner, 1944). In the case of the true Liesegang rings, however, a precipitation or chemical reaction is visible, whereas in the case of the observations reported herein, such rings remained invisible

until "developed" by allowing the organisms to grow.

MATERIALS AND METHODS

The organisms employed in the study were the following: *Micrococcus lysodeikticus* (Fleming strain), *Micrococcus lysodeikticus* (adapted strain originally obtained from Dr. Harold Wolin, Cornell University), *Bacillus subtilis* [ATCC 6633, the strain employed for subtilin production (Feeney and Garibaldi, 1948)], *Micrococcus pyogenes* var. *albus* (strain obtained from Overly Research Foundation), *Torulopsis utilis* var. *major* (ATCC 9256) and *Aspergillus niger* (NRRL 334).

All chemicals were of reagent grade unless otherwise specified. The 1,10-phenanthroline, 5-nitro-1,10-phenanthroline, 5-methyl-1,10-phenanthroline (5-meph), 4,7-dimethyl-1,10-phenanthroline and 2,9-dimethyl-1,10-phenanthroline were obtained from the G. Frederick Smith Co. The 8-hydroxyquinoline (oxine), 8-hydroxyquinoline-5-sulfonic acid, diphenylthiocarbazon and 8-hydroxy-7-iodo-5-quinoline-sulfonic acid were obtained from the Eastman Kodak Co. The benzoylacetone and 2-thenoyltrifluoroacetone were obtained from Graham, Crowley and Associates, Inc., and the ethylenediamine tetraacetic acid from the Bersworth Chemical Co. The 3,4-dichlorophenylserine was kindly supplied by C. M. Volkmann of the University of Texas. The conalbumin was prepared from egg white according to Feeney and Rhodes (1956); it was crystalline, electrophoretically pure by moving boundary analysis and was "free" of lysozyme. (By bacteriolytic assays these preparations were found to contain less than 0.001 per cent lysozyme.)

Medium A, a synthetic medium employed for *B. subtilis*, was similar to that described by Feeney and Garibaldi (1948). It differed from the

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latter medium in that it was employed at only half strength with one-fifth the amount of their Salt Mixture B. Medium P-2 was identical to medium P-2 previously described by Feeney and Nagy (1952), and consisted of 0.8 per cent nutrient broth (Difco), 0.50 per cent disodium phosphate and 0.03 per cent citric acid. Medium H, for cultivation of *M. lysodeikticus* (Wolin) consisted of 0.7 per cent glucose, 1.0 per cent glutamic acid, 0.2 per cent disodium hydrogen phosphate, 0.1 per cent ammonium chloride, 0.025 per cent sodium sulfate, 10 μ g per L biotin and the following cations as the chlorides in parts per million: K, 40; Mg, 5.0; Fe, 0.5; Zn, 0.5; Mn, 0.5; and Co, 0.5. The media employed for *A. niger* were similar to those of Sijpesteijn and Van Der Kerk (1956).

In conducting the studies in agar media, the following general techniques were employed: The media were constituted as described above; the pH was adjusted to the desired value (pH = 8.0 unless otherwise indicated); and 1.5 per cent agar was added. The agar was dissolved by heating and the pH of an aliquot was checked. Further additions of mineral elements, chelating agents, etc., were made. Petri dishes containing 20 ml of the sterilized and inoculated agar medium were prepared. The agar was allowed to harden approximately 30 min. The inoculum consisted of 0.5 ml of culture grown on the same liquid medium except in the case of *M. pyogenes* var. *albus* which was grown on nutrient broth (Difco). Filter paper discs approximately 1 cm in diameter were cut from Whatman No. 1 paper and sterilized separately in vials. Solutions containing the desired concentrations of the metals and reagents under study were added to the vials and the soaked paper discs were placed on the hardened agar. The dishes were then placed in the refrigerator at 2 C for 24 to 48 hr which allowed the materials under study to diffuse through the agar. The dishes were then removed and incubated at 37 C, except in the case of cultures of *A. niger* which were incubated at 27 to 30 C. In the later studies, as indicated in the text below, the inoculated dishes were incubated for 1 to 2 hr before placement of the discs and refrigeration.

RESULTS

Oxine-conalbumin interrelationships. In preliminary studies with *M. pyogenes* var. *albus* on

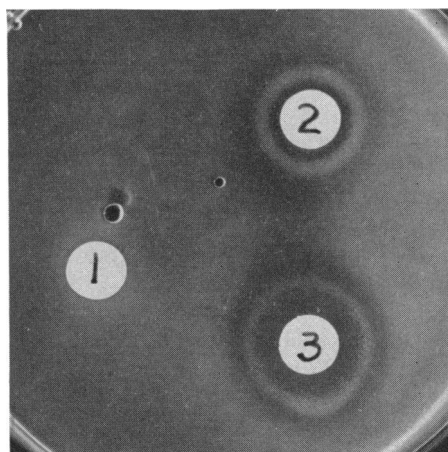


Figure 1. Rings of growth and inhibition of *Micrococcus pyogenes* var. *albus* with discs of 8-hydroxyquinoline on agar containing conalbumin. The discs labeled 1, 2 and 3 were saturated with solutions of 8-hydroxyquinoline at concentrations of 50, 100 and 200 μ g per ml, respectively. The concentration of conalbumin was 1.0 mg per ml. The medium was P-2 and the general procedure was as in the text. The incubation time was 20 hr and the temperature was 37 C.

medium P-2 inhibition rings approximately 25 mm in diameter were obtained with discs saturated with oxine solutions of 200 to 300 μ g per ml. When conalbumin was added to the medium in amounts approximately equivalent to the iron content (Fraenkel-Conrat and Feeney, 1950), the oxine discs usually gave several very distinct rings of growth and inhibition around the disc. Figure 1 is a photograph of a petri dish of one such experiment with 50, 100 and 200 μ g oxine. At the higher concentrations in this experiment there was a heavy ring of growth in the center of a larger ring of inhibition, and the relative sizes and positions of the rings were different with the two concentrations of oxine. In an attempt to obtain these alternate rings of growth with oxine in the absence of the conalbumin, thin and hazy rings were formed under certain conditions. These conditions required that the inoculated dish be incubated for 1 to 3 hr prior to placement of the discs and refrigeration. When this procedure was followed, as well as with the addition of conalbumin, the rings were usually multiple in nature. In one such experiment with conalbumin in the medium, the discs from 100 μ g

per ml oxine gave a total ring of inhibition of 45 mm with two well defined rings of growth of approximately 2-mm and 3-mm width within this larger ring of inhibition. It thus appeared that both intensification of the rings and the frequency of their occurrence were increased by a short incubation period after inoculation and before placement of the discs in refrigeration. The reason for this effect was not learned during this study.

Discs with various chelating agents. As a result of the apparent relationships between oxine and conalbumin observed in agar, a variety of chelating agents was tested with several different organisms on several different media. The effects were found to vary with the organisms, the media and the chelating agents in the following manner: no inhibitory action, inhibition rings without multiple ring formation and multiple alternate rings of growth and inhibition. The following chelating agents gave no rings of inhibition with *M. pyogenes* var. *albus* or *B. subtilis* on medium P-2 with discs placed in solutions of 500 μ g per ml: 3,4-dichlorophenylserine, 8-hydroxyquinoline-5-sulfonic acid, 8-hydroxy-7-iodo-5-quinolinesulfonic acid, diphenylthiocarbazone, 2-thenoyltrifluoroacetone, benzoylacetone and ethylenediamine tetraacetic acid. The majority of these same chelating agents gave no inhibition with *B. subtilis* on medium A, *A. niger* on the media of Sijpesteijn and Van Der Kerk (1956), *M. lysodeikticus* on medium P-3, *M. lysodeikticus* (Wolin strain) on medium H and *T. utilis* var. *maior* on medium P-2.

Of the various other agents tested, oxine, 1,10-phenanthroline and derivatives of 1,10-phenanthroline (5-methyl, 5-nitro, 4,7-dimethyl and 2,9-dimethyl) gave the most outstanding results. The best production of multiple rings was obtained with 5-meph (5-methyl-1,10-phenanthroline). The 2,9-dimethyl-1,10-phenanthroline, however, was strongly inhibitory towards all organisms tested, but no evidences of alternate rings of growth and inhibition were obtained under a variety of conditions. Oxine was only very poorly inhibitory for the yeast and for *A. niger*. Substituting nitrate or ammonia nitrogen for organic nitrogen in the media of Sijpesteijn and Van Der Kerk (1956) did not influence the type of rings formed with *A. niger*. Although multiple ring formation was obtained with several other organisms on the various

media, the further investigations of the present study were restricted to *M. pyogenes* var. *albus* and *B. subtilis* on medium P-2 because good multiple ring formation was obtained with both organisms on this one medium.

Discs with chelating agents on media containing metal ions. In a series of studies on this subject with *B. subtilis* grown on medium P-2 and on medium A and with other organisms grown on P-2, the degree of inhibition obtained by placing discs with various chelating agents on the agar was found related to the trace metal content of the media. The effect was primarily to increase or decrease the inhibition zones. For example, by the addition of cobalt to medium P-2 or to medium A the size of inhibition rings with oxine decreased with *M. pyogenes* var. *albus* or *B. subtilis*, while the addition of copper to the media caused the size of the rings to increase.

Discs with metal ions on media containing chelating agents. Similar, but more extensive effects were obtained when the chelating agents were placed in the media and the metal ions on the discs (the opposite relationship as described

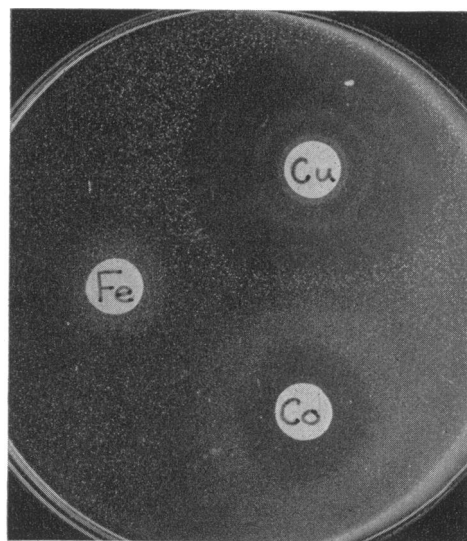


Figure 2. Rings of growth and inhibition of *Bacillus subtilis* with discs of metal ions on agar containing 1.25 ppm 5-methyl-1,10-phenanthroline. The discs were saturated with solutions containing 350 μ g per ml of the metal ions as indicated and 3.5 mg per ml of citric acid adjusted to pH 8.0 with sodium hydroxide. The medium was P-2 and the general procedure was as described in the text. The incubation time was 18 hr and the temperature was 37 C.

in the preceding section). The greater facility of this technique was primarily due to the ease in demonstrating the interplay of different metal ions. This facility, however, was also probably related to the greater mobilities of the metal ions as compared to the mobilities of the chelating agents. In general, the effects of copper, cobalt and iron were greater than those of other elements tested (zinc, manganese, nickel and molybdenum).

Figure 2 is a photograph of the effects of iron, copper and cobalt on the growth of *B. subtilis* on medium P-2 containing 2.5 ppm 5-meph. The difference in effects of the three elements is apparent. The fine rings of growth within the large area of inhibition were a general characteristic for *B. subtilis* on this medium and with this chelating agent. In the absence of the 5-meph, the metal ions did not influence the growth. In contrast, it was possible to obtain only one inner ring of growth around the copper disc surrounded by an outer ring of inhibition on the synthetic medium A with *B. subtilis*. During our studies, more than two rings were never obtained with *B. subtilis* on the synthetic medium.

The effects of concentration of chelating agent in the medium are seen in figure 3. Figures 3 A and 3 B are photographs of two dishes from one experiment on medium P-3 with *M. pyogenes*

var. *albus*. The media contained 1.25 ppm and 2.5 ppm 5-meph, respectively. These figures illustrate several points: (1) The relative effects of copper, cobalt and iron are readily seen. (2) Several types of inhibition may be obtained. In 3 A are results typical of many experiments in which there is a heavy area of growth around the copper discs with rings of partially reduced growth in this area and then a large area of inhibition at the outer edge. Another type of inhibition is merely a very large area of inhibition as shown by the copper in 3 B. Still a third type of effect is the production of the multiple alternate rings of growth and inhibition shown with the cobalt in 3 B. There are three rings of inhibition and two rings of growth giving a total number of five rings around the cobalt disc. (3) The concentration of chelating agent has a primary effect. The ring of heavy growth around the copper disc at 1.25 ppm 5-meph is absent at 2.5 ppm and the ring of inhibition is larger; however, the small ring obtained with cobalt at 1.25 ppm is replaced by large concentric rings of growth and inhibition at 2.5 ppm. Similar studies with oxine and *M. pyogenes* var. *albus* showed that the inhibition zones produced by copper would also increase in size as the oxine content of the medium was increased until the concentration was such that growth was completely inhibited. At this

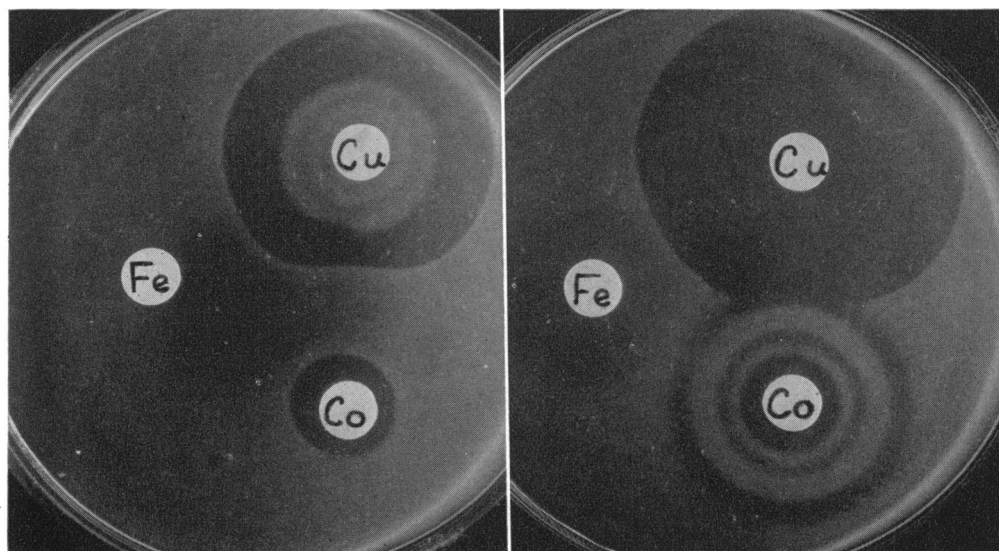


Figure 3. Rings of growth and inhibition of *Micrococcus pyogenes* var. *albus* with discs of metal ions on agar containing 1.25 and 2.5 ppm 5-methyl-1,10-phenanthroline. Left (A): 1.25 ppm 5-methyl-1,10-phenanthroline. Right (B): 2.5 ppm 5-methyl-1,10-phenanthroline. General conditions same as for figure 2.

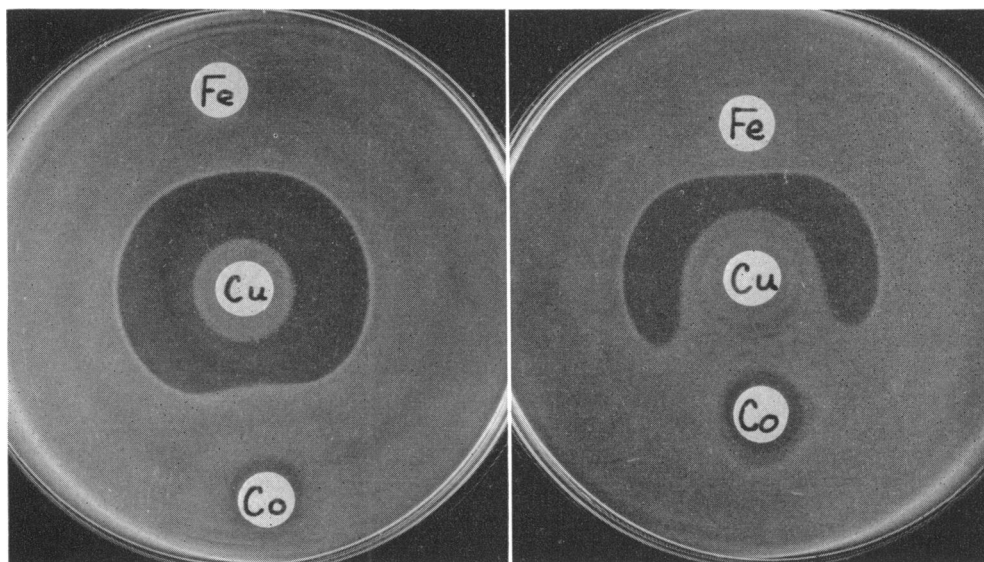


Figure 4. Rings of growth and inhibition of *Micrococcus pyogenes* var. *albus* with discs of metal ions on agar containing 1.25 ppm 5-methyl-1,10-phenanthroline. Influence of spacing of discs. Left (A): Discs approximately 3.0 cm apart. Right (B): Discs approximately 1.5 cm apart. General conditions as for figure 2 with the exception that the time of incubation was 25 hr.

concentration, a heavy zone of growth was obtained around the cobalt, whereas the copper and iron showed no effect.

By placing the discs containing the metal ions so that their diffusion areas would overlap, a variety of relationships was obtained. Figure 4 has photographs of two dishes from one experiment in which the distances between the discs containing the metal ions were varied. In this experiment, the medium was P-2 containing 1.25 ppm 5-meph and the organism was *M. pyogenes* var. *albus*. Figure 4 A shows an effect similar to that of figure 3 A at this same concentration of 5-meph. The copper has a large ring of inhibition surrounding an inner ring of heavy growth directly around the disc and the cobalt has a small ring of inhibition. In figure 4 B the discs are approximately twice as close to the copper disc as in figure 4 A. Here the cobalt area is strongly influencing the character of the copper area. An extension of this approach was to superimpose the discs containing metal ions on one another. When the metal ions, the order of their additions and the time intervals between these additions were varied, many different effects were obtained.

Varying the pH of the medium also gave pronounced effects. In most experiments the effects were similar to those obtained by varying the

concentration of chelating agent as shown in figures 3 A and 3 B. The nature of the effects, however, was dependent upon the particular chelating agent, the metal ion and the organism.

DISCUSSION

Our interpretation of the multiple rings of growth and inhibition which were obtained in this study is that the basic phenomenon is probably similar to that responsible for the production of Liesegang rings (Gortner, 1944). The cause of Liesegang rings remains unclear but the observations of the experimental facts show that certain salts will interact so as to produce a series of concentric rings or areas of interactions when one salt diffuses through a semisolid medium containing the other salt. The eye sees opaque or colored areas caused by chemical reactions and possibly chromatographic effects. In the studies reported in this paper no areas of chemical interaction were observable, but only the growth of the microorganisms. It is our opinion that the material (either the metal ions or chelating agents) placed on the disc diffuses through the medium and complexes with the chelating agent or metal ions in the medium to varying degrees depending upon the dissociation constants of the complexes and the relative concentrations. As the originally

added material and the newly formed complexes continue to diffuse, they would progressively equilibrate with the materials (ions or chelating agents) in the successive areas of the medium. There would thus be areas in which there are varying concentrations of the chelating agent, the different metal-chelate complexes and the free metal ions (similar to the Liesegang rings), but the relative concentrations and properties of the reactants would be such that they are not visible until "developed" by means of the growth of microorganisms.

The above interpretation is different from that of Sijpesteijn and Van Der Kerk who termed the areas of growth as "inversion growth." It does not appear necessary to postulate an inversion growth in areas where there is simply no inhibition of growth. According to our interpretation, the various concentric rings of growth and inhibition contain areas wherein the stoichiometric relationships of the chelating agent, different metal-chelate complexes and free metal ions are different. A ring of no growth is obtained in areas where the particular mixture is inhibitory. A ring of growth is obtained in areas where a different particular mixture is not inhibitory. Other factors such as the production of organic agents by the organisms might, of course, be of minor importance.

The results of this study may help explain the previously described interrelationships of conalbumin and oxine (Feeney, 1951; Feeney and Nagy, 1952). In these studies it was found that inhibitory concentrations of conalbumin and oxine were not inhibitory when tested as mixtures. This phenomenon appears similar to what one observes in the areas of growth within the concentric rings. Certain of the earlier studies of Rubbo *et al.* (1950) also appear to support our observations. In their studies it was reported that the inhibition of growth by oxine of some gram positive organisms decreased, increased and then decreased again upon successive purifications of the medium by extraction with fat solvents containing oxine. Oxine was inhibitory at very low concentrations on the original medium, whereas when the medium was extracted several times with oxine solutions, the inhibitory effect was greatly reduced. However, if the extractions were continued for several more times, the inhibitory activity of oxine was now as great as it was initially. Finally, if the extractions were continued

still further, the inhibitory action of oxine was again greatly reduced. This type of "shake-out" experiment appears very similar to what has been observed in the present studies when the chelating agent is diffusing from the disc through the medium and the metal ions and the chelating agents are progressively changing their concentrations. A similar relationship would also exist when the metal ions were on the discs and diffusing into the medium containing the chelating agent.

It is difficult to explain why many chelating agents inhibit microbial growth. Foremost among various investigators recently reporting on this subject are Albert and Rubbo and their co-workers (Albert *et al.*, 1953; Albert *et al.*, 1954; Rubbo *et al.*, 1950). These investigators explain that the inhibition of growth caused by oxine is due to the formation of toxic complexes of oxine, probably with copper or iron. According to their theory, cobalt prevents the inhibitory effect of oxine by preventing the action of the toxic complexes of copper or iron within the cell. The inhibition by oxine is thus not simply a result of a nutritional deficiency of cobalt. Feeney (1951) suggested that the effects may be a result of imbalances rather than the formation of a toxic complex, but the more recent results of Albert *et al.*, (1954) lend further support to the toxic complex concept. The very recent observation of Heath and Clark (1956) on the possible role of chelating agents in replacing the action of auxins in plants is of considerable interest in this regard. These authors reported that, whereas there is replacement of the auxin by a chelating agent, very low concentrations of one material in respect to the concentration of the other may give the opposite effect, i. e., inhibition. These observations suggest possible toxic mixtures such as might be present in the successive rings found in the present study.

Whatever may be the mode of action of the chelating agents and whatever may be the cause of the alternate rings of growth and inhibition, the results of the present study suggest that the effects obtained are intimately related to the relative concentrations of a variety of metal ions, the chelating agents, and the metal-chelate complexes. Further study of this phenomenon in agar media should give important information about the mode of action of chelating agents.

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SUMMARY

The formation of multiple alternate rings of growth and inhibition of growth of microorganisms on agar medium caused by chelating agents and metal ions are described and discussed. The rings were produced either by placing chelating agents in the medium and salts in filter paper discs on the surface of the medium or by placing the chelating agents in the filter paper discs. Concentric rings of growth and inhibition of growth were produced around the discs. Of the various chelating agents tested, 5-methyl-1,10-phenanthroline and 8-hydroxyquinoline gave the best formation of multiple rings with *Micrococcus pyogenes* var. *albus* and *Bacillus subtilis* as the test organisms.

The formation of the multiple alternate rings was compared with the formation of Liesegang rings caused by interactions of salts in gelatin or agar. The possibility that they are both caused by the same phenomenon is discussed.

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